A reversible smoke-induced secondary dormancy in Venus Flytrap (*Dionaea muscipula*) seed

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Abstract: The Venus Flytrap (VFT) is fire adapted because it regenerates vegetatively from its rhizome after seasonal fires. Many fire-adapted plants have seed that germinate following smoke exposure. Mature VFT seed are quiescent and germinate almost immediately when exposed to appropriate physical environmental factors. Here we show that smoke exposure induces secondary dormancy in VFT seed. Smoke-exposed seed exhibit either complete inhibition or significant delays of germination; however, the secondary dormancy can be reversed by soaking the seed in hydrogen peroxide solution or stratifying the seed. Whereas the treatment with peroxide resulted in germination comparable to controls, stratification resulted in germination over a prolonged time period. We propose induced dormancy demonstrates a unique fire adaptation as dormancy would prevent summer germination under adverse conditions following fire and stagger germination later in the fall or spring following winter stratification, allowing germination over a wider and more favorable range of environmental conditions.

Introduction

Historically, the Venus Flytrap (VFT) (*Dionaea muscipula*) was found associated with pocosin and longleaf pine semi-savannah of coastal North and South Carolina (Roberts & Oosting 1958). High quality longleaf pine (*Pinus palustris)* dominated habitats, and the herbaceous plants associated with them, require frequent low-intensity fires for population maintenance (Glitzenstein *et al.* 2001). The primary factors influencing the natural distribution of VFT were ascertained to be moisture and light intensity for localized populations, but overall distribution by soil type (Roberts & Oosting 1958), while fire is the dominant factor in maintaining populations (Gray *et al.* 2003). Although VFT evolved under a high-frequency fire regimen (Roberts & Oosting 1958), during fire-free periods plants are able to persist in a dormant state underground for an unknown period of time (Luken 2005). Highest flowering and seedling establishment occurs after fire although seedling survivorship is negatively impacted due to increased desiccation risk (Luken 2007).

While this plant has fascinated naturalists since its description in the mid-1700's, most studies concerning it have dealt with its carnivorous nature and the trapping mechanism of the modified leaves as well as the effect of varying fire regimens on VFT abundance, while few have dealt with demography. Studies specifically investigating VFT seed are virtually non-existent and the few studies that have been published, two of which are dated, address germination of the seed indirectly by investigating habitat and common environmental cues known to cause germination (Roberts

& Oosting 1958; Luken 2005; Smith 1931). The latter two studies did note the importance of fire in VFT habitat. Roberts and Oosting (1958) speculated that fire served as a means of eliminating competing vegetation; however, a later study (Luken 2007) found that simply removing competing vegetation did not enhance plant growth or new germination.

Observations reported in the popular press regarding VFT ecology may be contrary to those reported in peer-reviewed literature. One example is the supposition that VFT form seed banks (McPherson 2010). Short-lived species of carnivorous plants such as some *Drosera* and *Utricularia* may escape competition during fire-free intervals by the production of a persistent seed bank (Brewer 2001). Even though it is well known that seed viability diminishes over time (Schnell 2002), recent works still support the idea that seed banks develop from viable seed that remain dormant in the soil (Bailey & McPherson 2012).

Because it was not known if seed of VFT can accumulate in a persistent seed bank, we wished to investigate this possibility and whether they required any specific cues, particularly fire-related, to stimulate germination. As such, fresh seed and seed stored under a variety of conditions were exposed to different treatments under laboratory conditions. Treatments included stratification (6 weeks of exposure to moisture and cold [4°C] in the dark), soaking in hydrogen peroxide (H_2O_2), liquid smoke, hot water, and scarification.

Materials and Methods

Four cohorts of 1200-1500 seeds were used for our initial experimental protocol. Cohorts were based upon storage conditions: Cohort 1, comprised of seed collected June 2009 and immediately stored at 4°C until use within a few months (Fresh); Cohort 2, comprised of seed collected June 2008 and stored approximately 14 months at 4°C prior to use (Cold); Cohort 3, comprised of seed collected June 2008 and stored approximately 14 months at room temperature prior to use (Warm); and, Cohort 4, comprised of seed collected June 2008 stored at room temperature for approximately 13 months and then 4°C cold storage for 1 month (Warm/Cold). Subsequent experiments used freshly collected seed (July 2010) and 1-year-old seed that was stored at 4°C.

Seed from all 4 cohorts were subjected to up to 7 treatments prior to sowing (n=200 seeds/treatment) with direct sowing serving as a control. Sowing was done by scattering treatment groups between sheets of ddH_2O moistened Whatman #1 filter paper in 150 mm glass Petri dishes that were then sealed in clear plastic bags. All bags were placed in a Percival controlled environment chamber (Perry, IO) maintained at 25°C with a 14:10 L:D cycle under 155 µmoles/m^2/sec illumination.

There are several common methods for breaking dormancy of seeds that have been well researched. Fire-stimulated germination of seed has been postulated to include dry heat fracturing of the seed coat, stimulation of the embryo by dry heat, desiccation of the seed coat, and stimulation of germination by compounds found in the smoke (Brown 1993).

Another treatment that influences seed dormancy is cold stratification (moist chilling). Common cold stratification consists of keeping the seeds moist at low temperatures, generally 0-15°C. Ross (1984) and later Lewak *et al.* (2000) suggests that cold stratification initiates processes that allow the seed to utilize its nutrient storage. One of the first compounds broken down in stratification is phytic acid, which is stored as organic phosphates to be used as energy during germination (Andriotis *et al.* 2005). Eventually, cold stratification triggers proteins to break down into amino acids (Einali & Sadeghipour 2007). The amino acids are then utilized in construction of proteins used for germination (Rajjou *et al.* 2004).

Mechanical scarification breaks physical dormancy of seeds by weakening or fracturing the seed coat (Baskin & Baskin 2004), allowing the seed to imbibe water from the environment to serve

in germination (Pérez-Garcia & González-Benito 2006). Mechanical scarification may also aid in increasing oxygen uptake by the seed (Stabell *et al.* 1998). The effect of scarification is considered comparable to the cracks caused by extreme heat due to fire exposure (Herranz *et al.* 1998).

A modification of mechanical scarification is soaking seed in hot water. This treatment is commonly used in plants that have physical dormancy in which the seed coat hinders the entry of water. Turner *et al.* (2005) demonstrated that hot water could be an effective means of increasing the water absorption of several genera of *Rhamnaceae*. Most suggested mechanisms involve an anatomical change in the seed coat. For various species in the genus *Acacia*, hot water cracks the seed coat to allow water entry (Brown & Booysen 1969). In other plants, the anatomical change is a single crack near the emergence point of the radical caused by a small swelling at this location (Li *et al.* 1999). In both instances, the water-impermeable seed coat is broken to allow entry of water.

Recent studies suggest that H_2O_2 can stimulate germination. The mode of action was originally thought to be its disinfectant properties. Joseph *et al.* (1998) demonstrated that H_2O_2 limits the growth and proliferation of two species of the plant pathogenic fungus *Pseudocercospera* at very low concentrations. While limiting fungal growth would benefit the plant, the actual germination would not be stimulated by the H_2O_2.

Germination-inducing treatments consisted of 6 weeks of stratification; 24-hour soaking in hydrogen peroxide (1:2 v:v dilution of commercial strength solution in water) or liquid smoke (commercial food grade liquid smoke product [TRYME®Liquid Smoke, Reily Foods Company, New Orleans, LA] used at full strength or diluted 1:9 v:v in water); boiling water immersion; scarification; or direct sowing. The hot water treatment consisted of adding seeds to ddH_2O that been brought to a boil and allowed to cool until cessation of boiling. Seed were then added and left in the water as it cooled to room temperature for 24 hours. Scarification was performed on the day all other treatment seed were sown by placing seed into a 50 ml centrifuge tube with 5 ml of coarse sand. The tube was vigorously shaken for 5 minutes and the seed dispensed to a Petri dish as described. Some treatments were combined: exposure to smoke followed by exposure to H_2O_2 or followed by stratification. Germination was typically evaluated on a daily basis for 3 weeks after the first day of germination.

Log-linear analysis assessed the distribution of data from the cohort study by testing interactions of cohort, treatment, and germination. Hypothesis testing of partial independence and of conditional independence was performed following repeat analysis by means of a three-dimensional contingency table. Chi-square of contingency was utilized to compare treatment germination results within and between cohorts.

Replicate sowing data were analyzed by one-way ANOVA of seed treatment and age ($p=0.000$). Student Newman-Keuls was utilized as a post hoc ($p=0.05$).

An estimation of germination speed is given by /50 which indicates the time in which 50% of final germination was achieved (t^{50}). An ANOVA compared t^{50} values ($p=0.000$). A subsequent Games-Howell post hoc test was used to find homogenous groups within means. Differences between the treatments were considered significant at $p=0.05$.

The logrank test was used to compare distributions for the treatment effect. Kaplan-Meier curves were generated for the data to measure germination rate, using germination as the event of interest.

Results

Initial experiments examined storage conditions (cohort) and known germination inducers (treatment) on germination success. A three-way (cohort × treatment × germination) log-linear

Table 1. Log-linear analysis of known germination stimulants applied to *Dionaea muscipula* seed. Values represent percent (%) germination.

	Warm/Cold(%)	Cold (%)	Warm (%)	Fresh (%)
Liquid Smoke	0.00%[a]	0.00%[a]	0.00%[a]	0.00%[a]
Liquid Smoke, Dilute	N/A	68.50%[a, f]	N/A	71.00%[a, f]
Scarification	13.00%[a, b, A]	54.00%[a, b, f, A, B]	22.00%[a, b, A, B, C]	74.50%[a, b, A, B, C]
Stratification	17.50%[a, c, A]	57.00%[a, c, f, A, B]	6.00%[a, b, c, A, B, C]	0.00%[b, c, f, A, B, C]
Hydrogen Peroxide	33.50%[a, b, c, d, A]	71.50%[a, b, c, A, B]	20.00%[a, A, B, C]	59.00%[a, b, c, d, f, A, B, C]
Hot Water	21.50%[a, b, d, e, A]	64.50%[a, b, A, B]	16.00%[a, c, A, B, C]	38.50%[a, b, c, d, f, A, B, C]
Control	12.50%[a, d, e, A]	68.50%[a, b, c, A, B]	16.50%[a, c, B, C]	41.50%[a, b, c, d, f, A, B, C]

Treatments (n=200) were applied to four different cohorts of seeds: warm/cold, cold, warm and fresh. Treatments and cohorts were tested for significance by pair-wise comparison using a two-dimensional Chi-Square of Contingency. Lowercase superscript letters indicate statistical differences between treatments within cohorts. Uppercase letters indicate statistical differences between cohorts within treatments. The treatment or cohort that was used for a set of pair-wise comparisons is indicated as follows: a=Liquid Smoke, b=Scarification, c=Stratification, d=Hydrogen Peroxide, e=Hot Water, f=Liquid Smoke, Dilute, A=Warm/Cold, B=Cold, C=Warm.

analysis produced a final model that retained all effects, that there is significant interaction among all three variables in the population sampled. The likelihood ratio of this model was $X^2(0)=0$, $p=1$. This indicated that the highest order interaction (cohort × treatment × germination) was significant, $X^2(8)=39.18$, $p<0.001$. Analysis for partial independence of the cohorts determined that dependencies exist between all three variables. There was a significant association between the type of seed treatment and whether or not germination occurred. The results of subsequent Chi-square of contingency analysis of treatments within and between cohorts are presented in Table 1, and indicate significant pair wise differences between cohorts and between treatments within cohorts.

With two exceptions germination occurred in all treatments across all cohorts. One stratification treatment had no germination likely due to fungal contamination. All seed treated with full strength liquid smoke had complete germination failure. Across cohorts, all stratification treatments differed significantly, as was true for scarified, H_2O_2 and hot water exposed seed. Old seed stored at room temperature with or without a subsequent cold storage period had similar levels of germination that were significantly less than refrigerated or fresh seed, likely due to decreased viability. Within cohorts, seeds that had been stored refrigerated or at room temperature and then stratified germinated at a significantly reduced level versus controls. Fresh seed that had been scarified had improved germination than controls, possibly due to damage to the heavy walled outer seed coat and subsequent faster imbibition of water. H_2O_2 increases germination of fresh seed while hot water does not.

Across cohorts we observed obvious discrepancies in regards to germination success leading us to conclude that treatments were not breaking dormancy and that aged seed had, in fact, lost viability.

Because of the interactions noted from the cohort study and the observation during data collection that germination seemed to be occurring at different rates depending upon treatment, we ran new germination studies with replicates to allow for more in-depth analysis. ANOVA and post hoc analysis of replicated treatments (Table 2) confirmed that liquid smoke, an aqueous condensate of pyrolysis products released from controlled wood burning in the absence of air (Kim *et al.* 2011),

Table 2. Mean comparison of treatments of seeds with known germination stimulants using an ANOVA. Values are expressed as percent (%) germination.

	Mean +/- Standard Deviation (%)	
Treatment	Stored Seed	Fresh Seed
Liquid Smoke	26.0±17.4%[a]	35.0±12.8%[a]
Liquid Smoke/H_2O_2	25.0±7.6%[a]	86.0±5.2%[b]
Liquid Smoke, Dilute	35.0±16.8%[a]	84.0±9.8%[b]
Control	73.0±13.6%[b]	92.0±3.3%[b]
Stratified	74.0±18.6%[b]	86.0±8.3%[b]
H_2O_2	77.0±8.9%[b]	96.0±5.7%[b]
Liquid Smoke, Dilute/Stratification	–	85.0±10.0%[b]
Liquid Smoke, Dilute/H_2O_2	–	88.0±21.4%[b]

Stored seed was kept at room temperature for 14 months while fresh seed was utilized within 1 month of harvest. Each treatment is an average of 4 replicates (n=25). Treatments with forward slash (/) are combination treatments in which the seed was first treated with smoke and then treated with the second stimulus. Lowercase superscripts on means indicate significance groups based on a Student Newman-Keuls. Means in the same group are statistically different from means in the other group.

had an effect on germination. All seeds exposed to undiluted liquid smoke (hereinafter referred to as smoke) had significantly reduced final germination compared to all other treatments. While we anticipated smoke treated seed to have reduced viability based upon the cohort study, we were surprised to find that fresh seed was actually inhibited from germinating and not killed. Subsequent treatment of smoke exposed seed with stratification or H_2O_2 restored final germination to that of controls. Smoke apparently induces a secondary dormancy in fresh VFT seed.

As the post hoc analysis only examines final germination success to determine significance between treatments and ignores the rate of germination and germination time interval (Fig. 1), we analyzed germination speed (Fig. 2). Figure 1 illustrates how the rate of germination of the seed allowed us to appreciate that the different treatments do have an effect on germination not accounted for if only examining final germination results. Germination of fresh seed is extremely rapid, with almost 100% of viable seed germinating over 2 days, while smoke treated seed takes longer before initial germination and furthermore germination is prolonged over a longer duration of time. This inhibition of germination is reversed with either stratification or H_2O_2 treatment. Analysis of these rates by calculating a line of best fit and determining the slope is not possible because a straight line does not provide an accurate description of rate due to the unusual germination pattern of the seed.

The daily germination of seed over time (Fig. 2) graphically represents how smoke inhibits seed from immediate germination versus the rapid germination observed in the control. The few seeds that do germinate when exposed to smoke begin later and over a much longer period of time, with only 1 or 2 seeds germinating daily. Seeds inhibited by smoke and then treated by stratification or H_2O_2 show reversed dormancy. These seed begin to germinate around the same time as controls however their daily germination totals are lower than controls while the interval over which they germinate is prolonged.

Germination rates of control and dormancy reversed seed differ significantly from smoke inhibited seed (Fig. 3). Box and whisker plots do not account for censored data and the time to 50% germination can be identical for treatments that have differing germination rates and

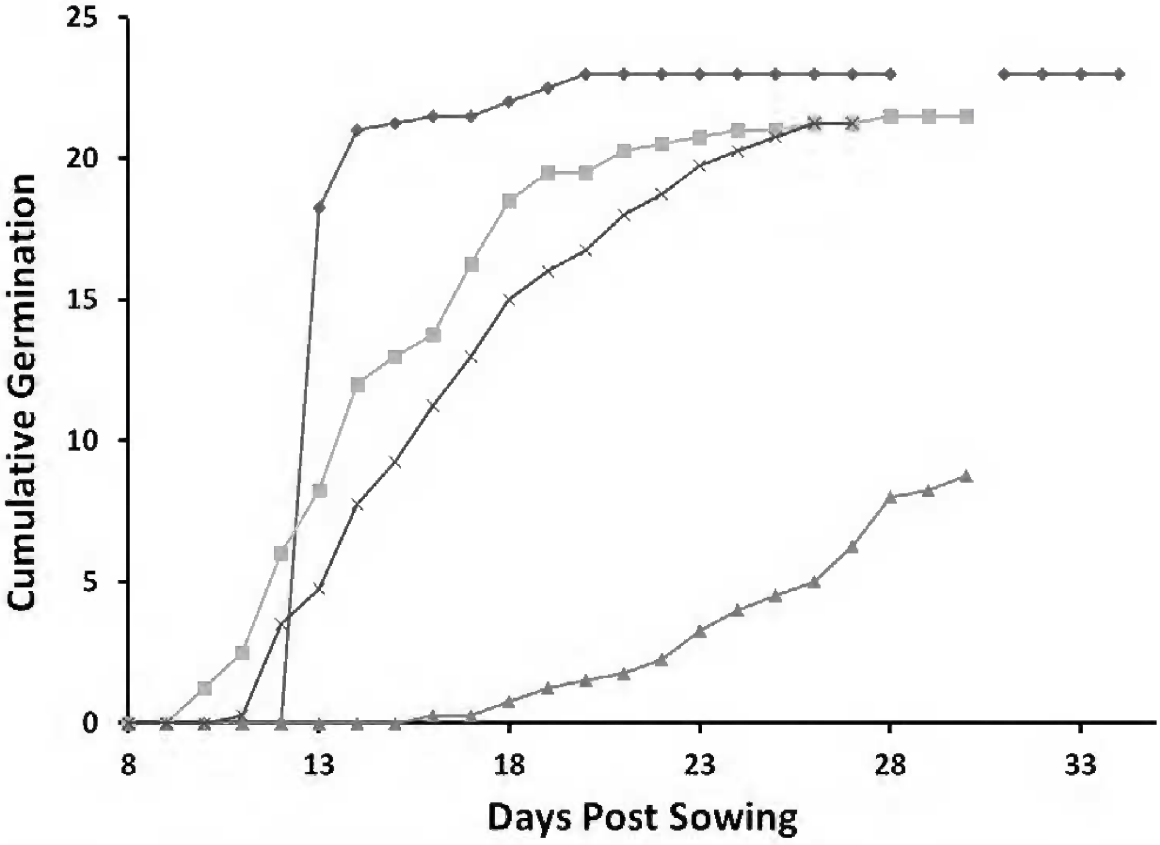

Figure 1: Effects of selected treatments on the rate of *Dionaea muscipula* seed germination. Each treatment is an average of 4 replicates (n=25). All germination studies for the selected treatments were conducted using immediately viable seed. Treatments with a forward slash (/) are combination treatments in which the seed was first treated with smoke and then treated with the second stimulus. Selected treatments are Control (—◆—), Liquid Smoke (—▲—), Liquid Smoke/H_2O_2 (—■—), and Liquid Smoke, Diluted/Stratification (—×—). The graphical representation of *D. muscipula* seed over time indicates that liquid smoke creates a secondary dormancy that is reversed by H_2O_2 and stratification.

significantly different total germination means. In addition, because 100% of the seed did not germinate for the control group or any of the treatment groups within the timeframe for monitoring germination, censored data (waiting time) resulted. To illustrate these differences, a Kaplan-Meier curve was constructed to show germination times for control, germination-inhibited seed induced by smoke treatment, and smoked-treated seed that was then treated with H_2O_2 or stratification to reverse the inhibition (Fig. 4). Here, the event of interest is germination. The germination curve for seeds inhibited with smoke is higher than the curves for the control and dormancy-reversed seed. Inhibition of germination is greater for the higher curve because the proportion of seeds that have not germinated is larger for this curve than for the lower curves at each time point. Similarly and more importantly, seed that was stratified to reverse smoke-induced inhibition took significantly longer than H_2O_2-treated seed to germinate ($p=0.02$) and H_2O_2-treated seed took significantly longer than the control ($p=0.01$). Kaplan-Meier graphing can illustrate trends that allows for discrimination among germination rates. From this we can resolve the discrepancy between scientific observations that no seed bank for VFT exists (immediate germination of ripe seed) and observations that suggest otherwise, such as a flush of germination following fire.

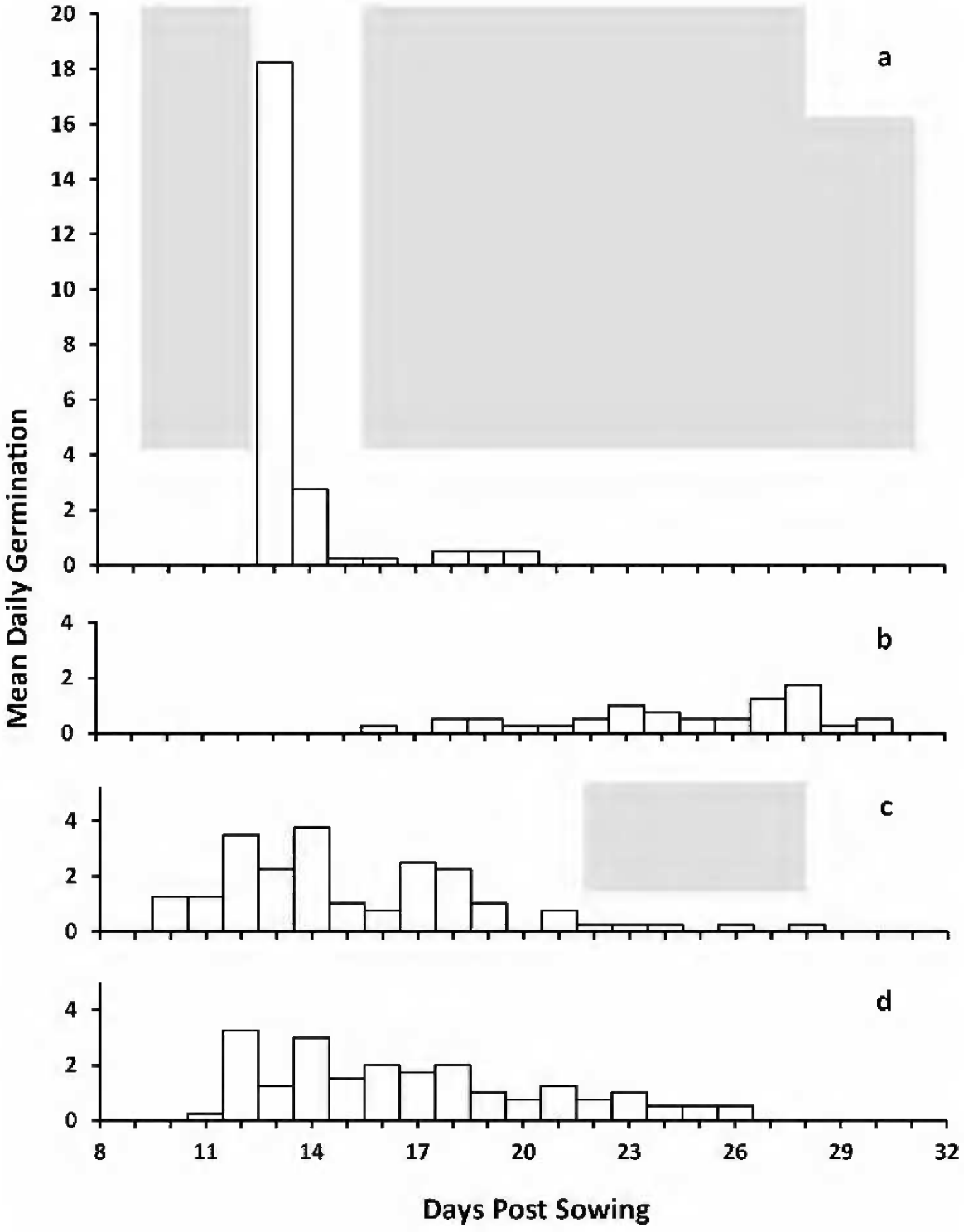

Figure 2: Treatment effect on daily germination of immediately viable *Dionaea muscipula* seed. Daily germination is the average of four replicates (n=25) for each treatment. The control treatment (a) indicates that *D. muscipula* seeds are immediately viable as shown by a large quantity of seeds germinating in a short period of time. Liquid Smoke (b) causes a secondary induced dormancy in seed indicated by few seeds germinating. Both combination treatments, Liquid Smoke/H_2O_2 (c) and Liquid Smoke, Diluted/ Stratification (d) indicate a reversal of dormancy due to most of the seeds within the treatment germinating.

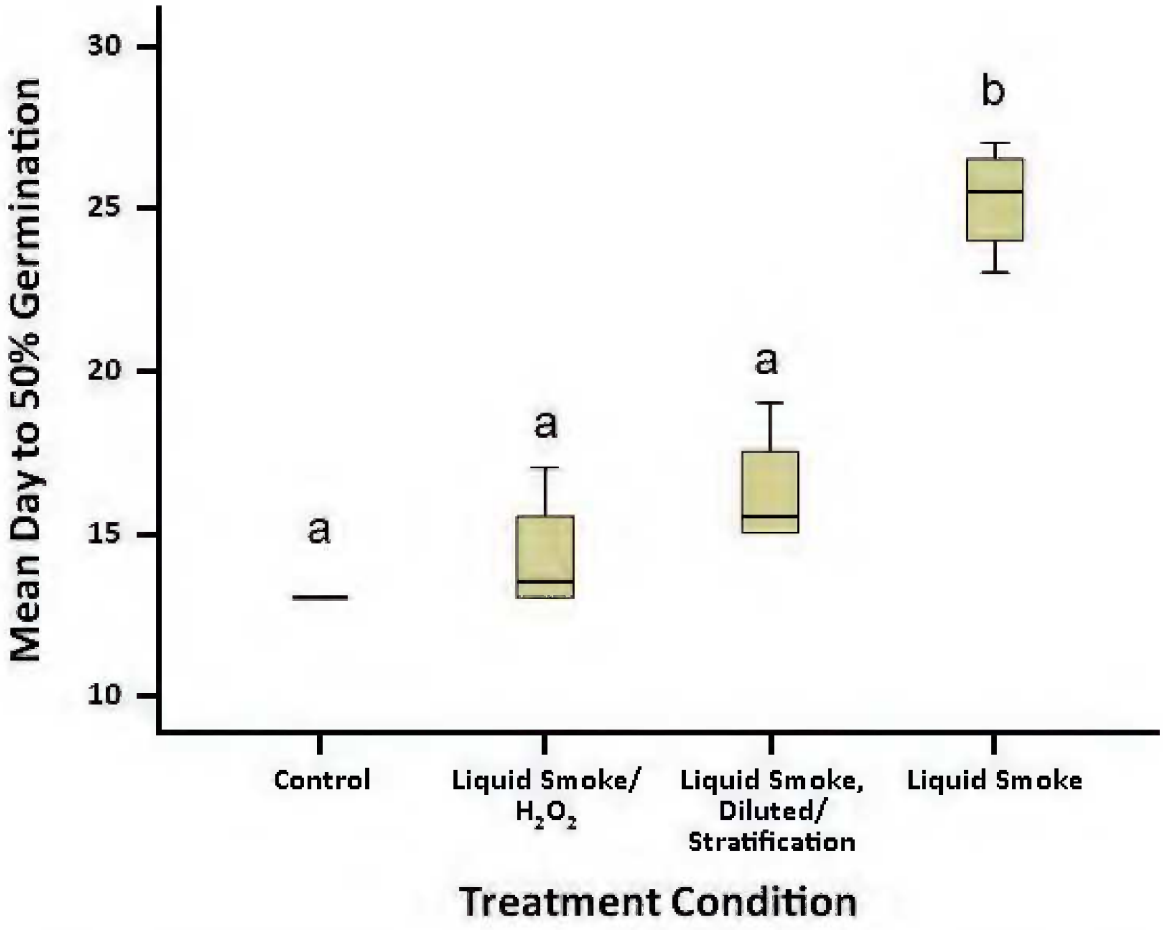

Figure 3: Analysis of germination rate of immediately viable *Dionaea muscipula* seed using time to fifty percent of total germination (t^{50}). For each plot, the box represents the interquartile range (25^{th} to 75^{th} percentile), the horizontal line within the box represents the median, and the bars represent the maximum and minimum distribution of the data. The t^{50} values were analyzed using an ANOVA to determine differences among treatments followed by a Games-Howell post hoc. Each treatment is an average of 4 replicates (n=25). Treatments with different letters are significantly different. Reversal of the inhibition of germination rate is reversed by either H_2O_2 or stratification.

Discussion

Studies have linked the chemical activity of smoke components to that of endogenous hormones in plants (van Staden *et al.* 2000). Todorović *et al.* (2005) demonstrated that when liquid smoke was applied to seeds of *Paulownia tomentosa* along with gibberellins, the hormones effectiveness was significantly increased. Actual components of smoke that elicit germination have been difficult to determine since smoke is composed of many different chemicals, some of which cause seed death if the concentration is too high (Nelson *et al.* 2009). Recently, 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one and analogous butenolide molecules designated as karrikins have been extracted from smoke and shown to provide germination cues for seeds (Nelson *et al.* 2009; van Staden *et al.* 2000).

Neill *et al.* (2002) suggests that H_2O_2 works as a signaling molecule, performing different functions in relation to concentration as demonstrated by Vandenabeele *et al.* (2003), with low *in planta* levels up-regulating genes (mostly associated with adverse environmental conditions) and high levels inducing programmed cell death. Dolatabadian and Modarres Sanavy (2008) showed that H_2O_2

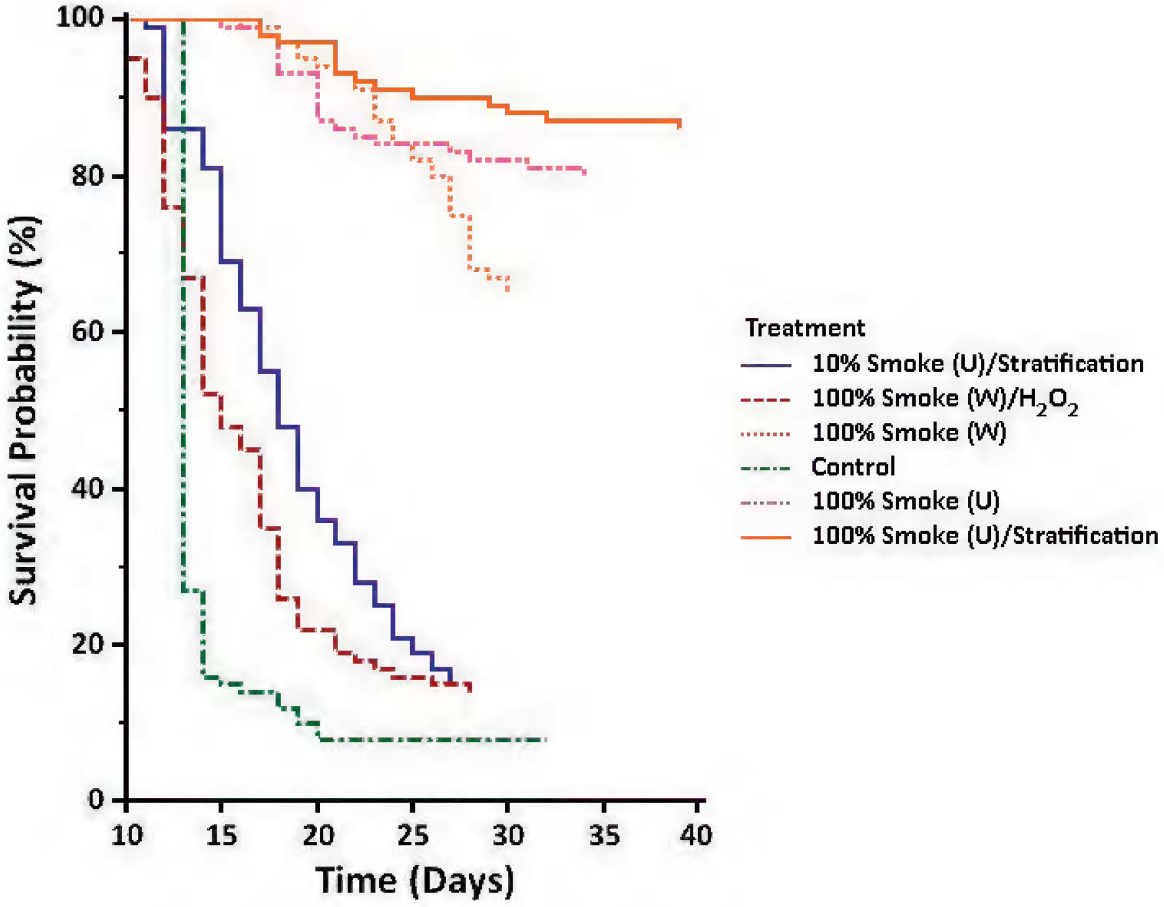

Figure 4: Kaplan Meier estimate of germination rate of immediately viable *Dionaea muscipula* seed. Each treatment is an average of 4 replicates (n=25). The germination probability is the likelihood of seed in the given treatment group not germinating. This probability can be interpreted to be a rate by analyzing change in germination probability over time. Survival curves were tested pairwise for statistical significance using a Chi-square goodness of fit test with $\alpha = 0.05$ for all tests. A "W" or "U" following the treatment description indicates the seeds were briefly washed (W) or unwashed (U) with distilled water following treatment.

positively influenced sunflower, rape, and safflower germination at lower concentrations (1 and 3%) due to germination inhibitor oxidation.

Because of the ability to germinate immediately upon ripening, seed of VFT do not accumulate in a persistent seed bank but may form a temporary seed bank. VFT seed, even though released from the plant in a non-dormant state, can be induced into a secondary dormancy by exposure to smoke from summer fires. In this manner, it is somewhat similar to "dormancy cycling" of some temperate annual species (Chen *et al.* 2011; Schütz 1998), preventing immediate germination during a favorable season of the year but under environmental conditions that are not favorable for seedling survival (Hilhorst *et al.* 2010; Luken 2007). As fire-free intervals may be critical for seedling survival and growth (Luken 2007), VFT seed exposed to smoke avoid germination and therefore a greater death risk from high temperatures and increased likelihood of desiccation. Germination will then occur later in the fall or the following spring after a period of moist stratification eliminates inhibiting substances from the seed (Light *et al.* 2002). Subsequent germination is spread over a much longer time interval than non-dormant seed. This prevents losses of seedlings that may have

germinated *en mass* during short periods of favorable conditions in the fall or spring, only to be killed by late or early frosts, respectively. Seeds that escape killing frosts would benefit from the increased light resulting from the fire-modified habitat as well as from the higher water table and increased rainfall, advantages not available during summer germination. Further investigation is warranted to determine if liquid smoke can be used to extend the viability of short-lived seed of agricultural importance.

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